IMPACT OF CHEMICAL MIXTURES FROM WASTEWATER TREATMENT PLANT EFFLUENTS ON HUMAN IMMUNE CELL ACTIVATION: AN EFFECT-BASED ANALYSIS

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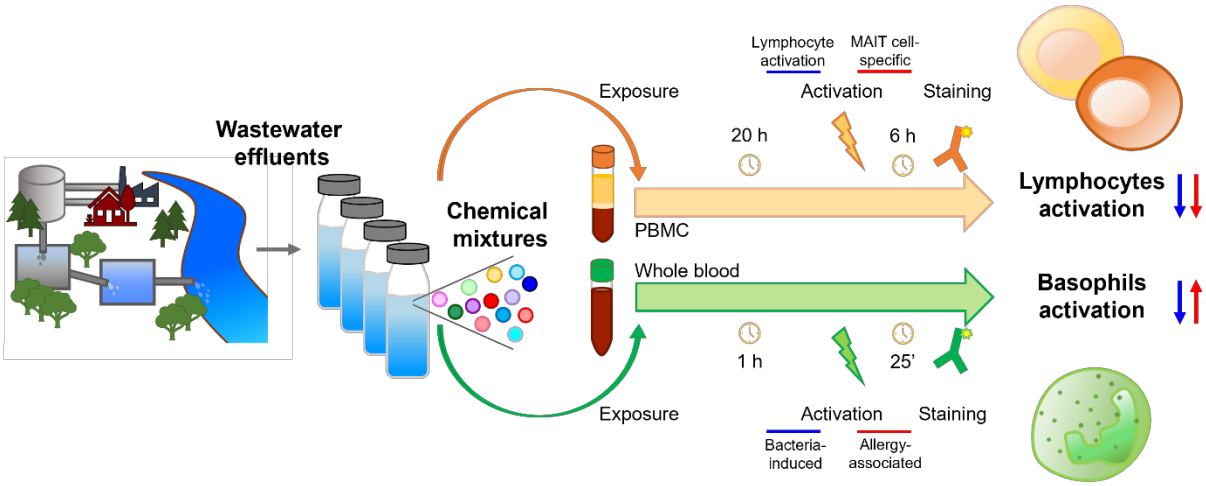
Graphical abstract



Abstract

Background: Humans are exposed to many different chemicals on a daily basis, mostly as chemical mixtures, usually from food, consumer products and the environment. Wastewater treatment plant effluent contains mixtures of chemicals that have been discarded or excreted by humans and not removed by water treatment. These effluents contribute directly to water pollution, they are used in agriculture and may affect human health. The possible effect of such chemical mixtures on the immune system has not been characterized.

Objective: The aim of this study was to investigate the effect of extracts obtained from four European wastewater treatment plant effluents on human primary immune cell activation.

Methods: Immune cells were exposed to the effluent extracts and modulation of cell activation was performed by multi-parameter flow cytometry. Messenger-RNA (mRNA) expression of genes related to immune system and hormone receptors was measured by RT-PCR.

Results: The exposure of immune cells to these extracts, containing 339 detected chemicals, significantly reduced the activation of human lymphocytes, mainly affecting T helper and mucosal-associated invariant T cells. In addition, basophil activation was also altered upon mixture exposure. Concerning mRNA expression, we observed that 12 transcripts were down-regulated by at least one extract while 11 were up-regulated. Correlation analyses between the analyzed immune parameters and the concentration of chemicals in the WWTP extracts, highlighted the most immunomodulatory chemicals.

Discussion: Our results suggest that the mixture of chemicals present in the effluents of wastewater treatment plants could be considered as immunosuppressive, due to their ability to interfere with the activation of immune cells, a process of utmost importance for the functionality of the immune system. The combined approach of immune effect-based analysis and chemical content analysis used in our study provides a useful tool for investigating the effect of environmental mixtures on the human immune response.

Keywords:

Chemical mixtures; wastewater treatment plant; immunotoxicity; lymphocytes; MAIT cells; effect-based analysis

Abbreviations:[1]

[1]Abbreviations:
B: blank
BAT: basophil activation test
BpC: bacteria per cell
DN: double negative
E: effluent (E1-E4)
EC: European community
EDCs: endocrine disrupting chemicals
FC: fold change
fMLP: N-formylmethionyl-leucyl-phenylalanine
IMDM: Iscove's modified Dulbecco's medium
LVSPE: large-volume solid phase extraction
MAIT: mucosal-associated invariant T
NA: not applicable
NK: natural killer
PBMC: peripheral blood mononuclear cell
PBS: phosphate buffered saline
REF: relative enrichment factor
RT: room temperature
SD: standard deviation
t-SNE: t-distributed stochastic neighbor embedding
WWTP: wastewater treatment plants

1. Introduction

The entire ecosystem and humans are constantly exposed to mixtures of potentially hazardous substances. More than 350'000 known chemicals have been registered in 2020 in the global market (Wang et al., 2020).

Although contaminating chemicals are usually present in low concentrations, their effects could be enhanced in mixtures. Many chemicals, including urban runoff chemicals, biocides and pesticides, personal care products, pharmaceuticals, and cleaning agents can pass the wastewater treatment plants (WWTP) and reach the water cycle as mixture of micropollutants with typical concentrations in the ng/L to µg/L range (Bourdat-Deschamps et al., 2014; Robles-Molina et al., 2014; Gorga et al., 2013; Ribeiro et al., 2015; Kümmerer, 2009). To date, these micropollutants are among the major contributors to aquatic pollution (Lee et al., 2022; Neale et al., 2020; Gago-Ferrero et al., 2020; Loos et al., 2013; Daughton, 2009). Even at such low concentrations, these compounds individually or as mixtures, may pose a toxic risk to aquatic life (Malaj et al., 2014), particularly due to the presence of pharmaceuticals (Yao et al., 2021; Wang et al., 2019; Giebułtowicz et al., 2018; Balakrishna et al., 2017; Ramos et al., 2016; Liu et al., 2015; Sim et al., 2010, Zhou et al., 2010; Kosonen and Kronberg, 2009; Daughton and Ternes, 1999). In addition to aquatic organisms, the chemical mixtures present in these effluents may also pose a risk to humans, due to treated wastewater reuse (Mukherjee & Chauhan, 2023). Currently, wastewater and treated wastewater irrigation is increasing to combat the depletion of freshwater resources and the water stress caused by climate change (Wu, 2020; Mishra et al., 2023; Dickin et al., 2016). This means that the general public could also be exposed, for example, by consuming food grown in contaminated areas or by bathing in contaminated recreational waters (Aftab et al., 2023; Khalid et al., 2018). Many publications show the chemical contamination of crops after irrigation with wastewater and treated wastewater, leading to contamination of the food chain (Othman et al., 2021; Xiaoqin Wu et al., 2014; Riemenschneider et al., 2016). For example, Xiaoqin et al. measured the levels of 19 commonly occurring pharmaceutical and personal care products in 8 vegetables irrigated with treated wastewater under field conditions showing a high detection frequency for caffeine, meprobamate, primidone, DEET, carbamazepine, dilantin, naproxen, and triclosan (Xiaoqin Wu et al., 2014). Malchi and colleagues (2014) estimated the health risk associated with consumption of wastewater-irrigated root vegetables by using the threshold of toxicological concern (TTC) approach. The data showed that the TTC value of for example lamotrigine can be reached for a child at a daily consumption of half a carrot (~60 g) (Malchi et al., 2014). The

use of untreated and treated wastewater to irrigate crops is common practice in arid and semi-arid regions, and climate change will expand these regions and the reuse of wastewater. As a result, and also because of globalization, it is likely that larger parts of the population will become dependent on potentially contaminated food. Thus, there is an urgent need for analysis of the impact of the chemical mix present in wastewater and sewage treatment plants on human health, especially at the level of the immune system, where alterations are at the root of the development of many diseases.

To date, only few studies have investigated the effects of chemical mixtures on humans (reviewed in Bopp et al., 2016; Kumari and Kumar, 2020; Mustafa et al., 2023). The most commonly used approach is to analyze the interaction with nuclear receptors such as aryl hydrocarbon, estrogen, androgen or glucocorticoid receptors (Vinggaard et al., 2021), but complex cellular test systems are missing in humans. Endocrine disruption has been identified as a major effect in waste and surface water (Brion et al., 2019; Carvalho et al., 2019; Gómez et al., 2021; Kase et al., 2018; Leusch et al., 2018; Kunz et al., 2017; Wernersson et al., 2015).

It is known that the endocrine and the immune system are closely linked (Wensveen et al., 2019; Greives et al., 2017) and that hormones can lead to the activation or suppression of immune cells. This can then trigger a tissue/organ and organism response. Furthermore, both innate and adaptive immune cells express hormone receptors (Buskiewicz et al., 2016) and can be directly targeted by chemicals. Endocrine-mediated changes in the immune system and immunomodulation by single chemicals have already been demonstrated (Maddalon et al., 2022a; Maddalon et al., 2022b; Krause et al., 2022; Adegoke et al., 2021; Rogers et al., 2013), but the effects of complex chemical mixtures on the immune system have rarely been investigated (Bulka et al., 2021; Warner et al., 2021; Bonefeld et al., 2017; Thompson et al., 2015). The few data available suggest an immunotoxic effect of chemical mixtures (O'Dell et al., 2021; Maddalon et al., 2023), but the studies are limited to a small number of chemicals tested. It is important to study the effect of chemical mixtures on the immune system because increased activation of the immune system can lead to autoimmune diseases or allergies, while decreased activation (e.g. immunosuppression) can increase the risk of infection or neoplasia, both of which pose a risk to human health (Rijkers et al., 2021).

Our present investigation, building on studies by Finckh and colleagues (2022a; 2022b), integrates the characterization of four European WWTP effluent samples into an immune-effect-based analysis. We investigate the effect of chemical mixtures present in WWTP effluents on the activation of human immune cells, namely T helper ($CD4^+$), cytotoxic T

(CD8$^+$), mucosal-associated invariant T (MAIT), natural killer (NK), NKT cells, B cells, and basophils. These cells cover both the innate and the adaptive immune response. With this study, we want to point out the need to test the putative health effects, particularly at the level of immune cells, of treated wastewater prior to its reuse in order to identify chemicals of concern, and to improve the quality of wastewater treatment.

2. Methods

2.1. Preparation of WWTP effluent extracts

From a set of 56 European WWTP effluent samples (Finckh et al., 2022a; 2022b), four of them were selected based on the overall chemical burden (e.g. including EDCs) and *in vitro* PPARγ and aryl hydrocarbon receptor responses previously obtained on SH-SY5Y cell line (Lee et al., 2022). WWTP effluents were sampled in four different European countries between summer 2018 and spring 2019 as specified in Table 1. Sampling, extraction and storage of the WWTP effluents has been previously described by Finckh et al. (2022b). In brief, WWTP effluents were extracted on site using a large-volume solid phase extraction (LVSPE) device with a polymer sorbent (Macherey-Nagle HR-X). For the preparation of blank samples, 1 L of liquid chromatography mass spectrometry grade water was extracted by LVSPE in the laboratory, transported during sampling and processed along with the sample cartridges. After elution, the extracts were dissolved in methanol at a relative enrichment factor (REF) of 1000 and stored at -20 °C until further analysis. The concentrations of the chemicals detected by LC-HRMS screening in the four WWTP extracts are shown in Supplementary File 1 (reprinted from Finckh et al., 2022b). For simplicity reasons, from now we name the WWTP effluent sample extracts "WWTP extracts".

Prior to cellular treatment, WWTP extracts were thawed, and methanol was evaporated under nitrogen. The extracts were resuspended in the same volume of culture medium (IMDM or BAT buffer – explained below) to obtain the enrichment factor of 1000 compared to the original WWTP extracts. Several dilutions were tested (REF 25, 12.5, and 6.25).

2.2. Peripheral blood mononuclear cells (PBMCs) treatment with WWTP extracts

The study was approved by the Ethics Committees of the University of Leipzig (#079-15-09032015). Buffy coat blood from five pseudonymous healthy male donors was obtained from

the blood bank at the University of Leipzig, after written informed consent. Only male donors were selected to avoid intra-cycle differences among women. PBMCs were isolated by density gradient centrifugation, using Ficoll-Paque Plus (Cytiva Sweden AB, Uppsala, Sweden) and PBMCs were stored at -150 °C in FCS/10%DMSO until use. For experiments, PBMCs were thawed and suspended in cell culture medium, IMDM (Iscove's modified Dulbecco's medium - Gibco, Thermo Fisher Scientific, Waltham, US), supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, US), 1X Penicillin-Streptomycin (Gibco, Thermo Fisher Scientific, Waltham, US), and 50 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, US). PBMCs were seeded at $1x10^6$ cells/well in 96-well U-bottom microplates (Greiner Bio-One, Frickenhausen, Germany) and rested for at least 2 hours at 37 °C in a 5% CO_2 incubator before treatment.

PBMCs were exposed to WWTP extracts at final REF of 6.25, 12.5, and 25. Initial experiments proved that a final REF of 25 did not exhibit cytotoxicity on stimulated PBMCs (Supplementary Figure 1A), and concurrently to each experiment, cell viability was assessed for each exposure condition. As control for the WWTP extracts one column elution sample (B – EU203) was used as blank at a REF of 25. After 20 h exposure at 37 °C and 5% CO_2, PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies (anti-CD3/CD28) or with *Escherichia coli* K12 (*E. coli* K12) for 6 hours, as better explained in the next paragraphs. Negative controls were included, namely PBMCs stimulated with anti-CD28 alone and with IMDM only, respectively. Stimulation with anti-CD28 allows to evaluate unspecific activation levels. None of the donors showed unspecific activation, therefore all donors were considered suitable for the analyses.

2.2.1. Lymphocytes activation

Lymphocytes activation was induced by activating CD3 and CD28 molecules expressed on lymphocytes. Soluble anti-CD3 (clone: OKT3; 0.5 ng/mL) and anti-CD28 (clone: CD28.2; 0.5 μg/mL) (BioLegend, San Diego, US) antibodies were added to PBMCs previously exposed to WWTP extracts and incubated for 6 hours at 37 °C and 5% CO_2. The optimal concentrations of anti-CD3 and anti-CD28 were previously determined, and the expression of the activation markers induced by anti-CD3/CD28 stimulation are reported in Supplementary material (Suppl. Fig. 2 for $CD4^+$ and $CD8^+$, Suppl. Fig. 3 for MAIT and NKT cells, and Suppl. Fig. 4 for B, NK, and double negative – DN cells). To block cytokine secretion, Brefeldin A (10 μg/mL; Sigma-Aldrich, St. Louis, US) was added for the last 4 h of incubation.

2.2.2. MAIT cells specific activation

To specifically activate the MAIT cells, a T cell subtype reacting to bacterial metabolites, an activation with bacteria was used. The bacterial strain *Escherichia coli* K12 MG1655 was cultivated as previously described (Krause et al., 2022) and stored at -80 °C until use. Following 20 hours of exposure to WWTP extracts, PBMCs were stimulated with 10 BpC (bacteria per cell) of *E. coli* K12 for 6 hours at 37 °C in a 5% CO_2 incubator, and for the final 4 hours of incubation, Brefeldin A (10 µg/mL) was added (Suppl. Fig. 5 shows the degree of MAIT cell activation).

2.3. Flow cytometrical analysis of PBMCs

Following stimulation, PBMCs were transferred to V-bottom 96-well microplates (Thermo Fisher Scientific Waltham, US). Dead cells were excluded by staining with fixable viability dye-Zombie NIR™ (BioLegend) for 15 minutes at RT. Staining for surface markers (CD3, CD4, CD8, CD19, CD56, CD161, and TCRVα7.2, Suppl. Table S1) was performed for 20 minutes at RT. After staining and washing, PBMCs were fixed using FACS™ Lysing Solution (BD Biosciences, San Jose, US) for 10 minutes, and permeabilized with FACS™ Permeabilizing Solution 2 (BD Biosciences, San Jose, US) for further 10 minutes. Finally, cells were stained for intracellular markers (CD69, CD71, CD134, CD137, TNF-α, and IFN-γ, Suppl. Table S1) for 20 minutes at RT. The cytometric analysis was performed using the flow cytometer Cytek Aurora (Cytek Biosciences, California, US). A minimum of 100,000 viable T cells (NIR^-CD3^+) were acquired per sample, then lymphocytes were identified among total PBMCs using FSC-A and SSC-A. The gating strategies for lymphocytes and MAIT cells are reported in Suppl. Fig. 6. Briefly, lymphocytes were detected, following stimulation with anti-CD3/CD28, as follows: regarding $CD3^+$ cells, T helper cells were gated as $CD3^+CD56^-CD161^-TCRV\alpha7.2^-CD4^+$, cytotoxic T cells were gated as $CD3^+CD56^-CD161^-TCRV\alpha7.2^-CD8^+$, double negative (DN) were gated as $CD3^+CD56^-CD161^-TCRV\alpha7.2^-CD4^-CD8^-$, NKT cells as $CD3^+CD56^+$, and MAIT cells as $CD3^+CD56^-CD161^+TCRV\alpha7.2^+$; for $CD3^-$ cells, instead, B cells were gated as $CD3^-CD4^-CD19^+$, and NK cells as $CD3^-CD4^-CD56^+$ (Suppl. Fig. 6A). Regarding the specific activation of MAIT cells, with *E. coli* K12, MAIT cells were gated as $CD3^+CD8^+CD161^+TCRV\alpha7.2^+$ (Suppl. Fig. 6B). The expression of activation markers and intracellular pro-inflammatory cytokines within these lymphocyte populations were analyzed individually for TNF-α, IFN-γ, CD69, CD71, CD134, CD137, and the double expression of TNF-α and CD69 were expressed as % of positive cells for $CD4^+$, $CD8^+$, NKT, MAIT, $CD4^-CD8^-$, NK, and B cells. Only the activation markers responding to the stimulus in the certain

immune cell populations are shown. Data analysis was performed using FCS Express 7 (De Novo Software).

2.3.1 t-SNE analysis of stimulated PBMCs

Following conventional flow cytometrical analysis, an in-depth analysis for high dimensional data using t-distributed stochastic neighbor embedding (t-SNE) transformation tool (FCS Express 7, De Novo Software) was performed on gated $CD4^+$ cells following anti-CD3/CD28 stimulation and on gated MAIT cells following *E. coli* K12 stimulation. These cells were chosen because the effect of WWTP extracts on them was most pronounced. As representative effect, the highest tested concentration of every WWTP extract at REF of 25, together with the blank were selected for each donor. For the t-SNE analysis, the samples were merged and gated as for conventional analysis – Suppl. Fig. 6A and 6B. Briefly, 50,000 total events for $CD4^+$ cells and of 200,000 for MAIT cells were selected. The down-sampling was selected as interval, with iteration number: 500, perplexity: 50, and approximation: 0.5. Furthermore, the opt-SNE and the estimation for unsampled events were chosen to generate 2D t-SNE maps. The single treatment conditions (5 per donor) and the group conditions (B, E1, E2, E3, E4) were gated through the use of sample ID. Clusters of cells based on the expression level of the activation markers and of the cytokines were manually created, and the % of gated cells in each cluster was further analyzed.

2.4. Basophils activation

To activate basophils, following exposure to WWTP extracts, whole blood was stimulated in two different ways, to elicit both innate and adaptive response. Heparin-blood from eight male healthy pseudonymous volunteers was obtained from the blood bank at the University of Leipzig, after written informed consent (#079-15-09032015). For basophil activation, more blood samples were used than for lymphocyte activation experiments to provide more robust data due to the high variability in human basophil counts and reactivity. 100 µL of blood were transferred into flow cytometrical 5 mL polystyrene round-bottom tube (Falcon, Corning, New York, US) and exposed to the four WWTP extracts at a REF of 25 or to the blank that were previously resuspended in BAT buffer (MgCa PBS buffer supplemented together with IL-3; 2 ng/mL – Sigma-Aldrich, St. Louis, US). In parallel, as a negative control, blood was exposed to BAT buffer. Again, cell viability was assessed concurrently with each experiment, (Suppl. Fig. 1B). After 1 h incubation at 37 °C in a 5% CO_2 incubator, the basophils contained in the

whole blood were activated by the addition of anti-FcεR1α antibody (0.1 µg/mL, BioLegend) or N-formylmethionyl-leucyl-phenylalanine (fMLP, 0.05 µM, Sigma-Aldrich, St. Louis, US). Cells were stained with antibodies anti-CCR3 and anti-CD63 (Suppl. Table S2) for 25 minutes at 37 °C in a 5% CO_2 incubator. After 25 minutes, the reaction was stopped by adding EDTA 3.8% (Gibco, Thermo Fisher Scientific, Waltham, US), and erythrocytes were lysed by incubating the cells in erythrocytes lysis buffer (NH_4Cl – Sigma-Aldrich, NaCO3 – KMF Laborchemie, Lohmar, Germany, EDTA – Thermo Fisher Scientific) for 10 minutes at RT (twice). To discriminate between live and dead cells, cells were stained with fixable viability dye-eFluor™ 506 (eBioscience) for 20 minutes at 4 °C. Cells were fixed in paraformaldehyde 1% (Sigma-Aldrich) and analyzed on a FACS Canto™ II instrument (BD Biosciences, San Jose, US). Leukocytes were identified using scatter properties FSC-A and SSC-A, basophils were identified as $CCR3^+$ cells. The level of basophil activation was analyzed in $CCR3^+$ population as % $CD63^+$ cells and subdivided in CD63 high and low (the gating strategy is shown in Suppl. Fig. 7A). The basophil activation levels are reported in Suppl. Fig. 7B. Data were then analyzed using FCS Express 7 software.

2.5. Gene expression analysis (mRNA)

PBMCs were plated at a concentration of $1x10^6$ cells/well in IMDM and exposed to WWTP extracts at a REF of 25 or to the blank for 20 hours at 37 °C in a 5% CO_2 incubator. In order to specifically target the activation of MAIT cells, which were highly affected by WWTP extracts exposure, PBMCs were then stimulated with E. coli K12 (10 BpC) for 6 hours at 37°C and 5% CO_2. After the treatment, PBMCs were transferred to 1.5 mL microfuge tubes and centrifuged. PBMCs were suspended in 300 µL Trizol (Invitrogen, Waltham, US) and maintained at -80 °C prior to RNA extraction. Total RNA was extracted according to manufacturer's instructions (Invitrogen). cDNA synthesis was carried out with 200 ng of RNA by using 5U RevertAid™ H Minus Reverse Transcriptase (Fisher Scientific, Schwerte, Germany). Intron-spanning primers were designed and UPL probes were selected by the Universal Probe Library Assay Design Center. A number of 40 genes of interest and 3 reference genes (Suppl. Table S3 and S4) were pre-amplified in 12 cycles and quantitative PCR was performed on a 96x96 Dynamic array with BioMark™ HD System (Fluidigm, München, Germany). The cycling program consisted of 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR was performed with FastStart Universal Probe Master Mix (Roche, Mannheim, Germany). The analyzed genes were chosen according to their involvement in the immune system, in particular

on MAIT cells activation and functionality. Furthermore, some hormonal receptors were chosen.

2.6. Correlation between chemical composition and immune endpoints

The correlation between chemical concentrations of WWTP extracts and relative immune endpoints (lymphocytes activation, gene expression, and basophils activation) was evaluated using Kendall correlation method for non-normal distributed samples using the Corrr package in R and corrplot for P-value extraction. Correlation coefficients were visualized using the heatmap.3 function. Details are presented in Suppl. File 2.

2.7. Statistical analysis

Flow cytometric data were shown as fold change (FC) relative to the blank control. Statistical analysis was performed using GraphPad Prism (version 9.4.0). Data were reported as mean of 5 donors regarding lymphocytes and as mean ± standard deviation (SD) of 8 donors for basophils. Gene expression data were normalized to the average of the three reference genes and then to the minimum of the expression of each gene. FC was calculated on the blank. Normal distribution was assessed using the Shapiro-Wilk test. To calculate differences between treatments, one-way ANOVA, followed by Dunnett's multiple comparison test for several enrichment factors or ratio paired t-test for one enrichment factor were calculated. Unpaired t-test with Welch's correction was performed for gene expression analysis. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. WWTP extracts reduce the activation of lymphocytes upon anti-CD3/CD28 stimulation

PBMCs were exposed to increasing REF of WWTP extracts and then stimulated with anti-CD3/CD28 to induce the activation of $CD4^+$, $CD8^+$, NKT, MAIT, and $CD4^-CD8^-$ (DN) cells. The stimulus can also induce $CD3^-$ lymphocytes activation indirectly, through the activation of T cells. In this case, we refer to B and NK cells. None of the WWTP extract concentrations used had an effect on cell viability (Suppl. Fig. 1). WWTP extracts reduced the activation of different T cell populations (Figure 1). In particular, the activation of $CD4^+$ (Fig. 1A), MAIT

cells (Fig. 1C), and $CD8^+$ cells activation was decreased (Fig. 1B), whereas no concentration-dependent effect could be observed for NKT cells (Fig. 1D). This indicates in general a reduction of cells activation by all WWTP extracts, except E1 (REF 12.5) which was able to increase the expression level of TNF-α and TNF-α-CD69 in $CD8^+$ and NKT cells. WWTP extracts E2, E3, and E4 reduced T cell activation already at the lowest concentration (REF 6.25), therefore potentially reducing T cell activation without enrichment, at the real concentration found in the effluent. In contrast, WWTP extract E1 at the lowest REF mostly affected MAIT cells and to a lower extent $CD4^+$ cells. Finally, all WWTP extracts reduced T cells activation, with a clear concentration-dependent response.

Regarding the other $CD3^+$ subpopulation, $CD4^-CD8^-$ cells, which resemble with high probability γδT cells (Ribot et al., 2021), WWTP effluents also decreased the activation of these cells, mainly acting on CD137 (Suppl. Fig. 8C). Instead, regarding $CD3^-$ lymphocytes, they also induced a decrease in NK and B cells activation (Suppl. Fig. 8A and B) already at the lowest concentration, indicating that the immunomodulating effect of the WWTP extracts was not exclusive on T cells.

As $CD4^+$ and MAIT cell activation was strongly affected by exposure to the chemical mixtures in the analyzed WWTP extracts, a detailed t-SNE analysis was performed for these cell populations (Fig. 2 and 4). Regarding $CD4^+$ cells, 17 clusters of activated cells were identified in the map containing the merged donor and treatment conditions (Fig. 2B). The colors and the % of cells in these clusters are shown in the table of Fig. 2B. Note, that due to low cell numbers in some clusters and overlap, not all colors are visible in the 2D t-SNE map. All of these activated $CD4^+$ subpopulations were decreased by the exposure to WWTP extracts (REF 25), with a stronger effect from E1 to E4, as shown in the corresponding heat map (Fig. 2C) and exemplary 2D t-SNE maps for the expression of CD134 and CD137 (Fig. 2A). The maps for TNF-α, CD69, and CD71 expression are presented in Suppl. Fig. 9. More in depth, two highly affected cell populations were identified, and they are highlighted in red and blue dotted ovals in Fig. 2B. The most targeted population (red circle) includes mainly cells represented in cluster 2 of TNF-α, cluster 2 of CD134 and cluster 4 of CD137. The blue dotted population consists mainly of cells shown as cluster 1 TNF-α, cluster 1 CD134 and cluster 1 CD137. Cluster 1 of CD69 and of CD71 present some cells in both highly targeted populations, marked with violet circles in Fig. 2C. From Fig. 2C it can be seen that the two highly affected cell populations consisted of cells that were positive for the majority of activation markers. Thus, the selected

WWTP extracts have a preferential effect on highly activated cells, and not on cells expressing only few activation markers.

3.2. WWTP extracts reduce the activation of MAIT cells upon E. coli K12 stimulation

Since we observed that MAIT cells were one of the most targeted immune cell populations, PBMCs were stimulated with *E. coli* K12 to further investigate the effect of WWTP extracts specifically on the activation of these cells. MAIT cells are T cells involved in the first defense to bacteria (Legoux et al., 2020). They have been found to be susceptible to several chemicals, such as bisphenols and perfluorinated substances (Krause et al., 2022; Maddalon et al., 2023). With the stimulation of MAIT cells by *E. coli*, the immunosuppressive effect of WWTP extracts was even more pronounced (Fig. 3B). For E3 and E4, the inhibitory effects were observed already at the lowest concentration, whereas WWTP extracts E1 and E2 exhibited inhibitory effects on MAIT cell activation at REF of 12.5 (Fig. 3A). Again, a clear dose-dependent immunosuppression was observed for all WWTP extracts.

MAIT cells were also further analyzed using t-SNE dimensionality reduction (Fig. 4A), as they were among the cells mainly affected by WWTP extracts exposure, as were $CD4^+$ cells. Different clusters of activated MAIT cells were identified (Fig. 4B), all of which were decreased by WWTP extract (REF 25) exposure (Fig. 4C). In parallel, the percentage of low activated and inactive cells (grey, Fig. 4A and purple and violet in Fig. 4B) was highly increased after treatment with WWTP extracts (Fig. 4C). Notably, TNF-α cluster 2, IFN-γ cluster 1 and CD69 cluster 1 were composed of the same cells (Fig. 4B, red dotted circle) and represent highly activated MAIT cells expressing all three activation markers. Thus, these cells represent the most affected population, showing the highest immunosuppressive effect upon exposure to WWTP extract. The second active population is represented by cluster 1 TNF-α^+ and cluster 2 $CD69^+$ (Fig. 4B, green dotted oval) and represents the second population of MAIT cells whose activation was highly reduced.

3.3 Transcript analysis of PBMCs activation upon E. coli K12 stimulation

Since MAIT cells were among the most severely suppressed immune cells, we investigated the effects of WWTP extracts at a REF of 25 on MAIT cell mRNA expression using the specific stimulation of PBMCs with *E. coli*. Among the 40 genes analyzed, the mRNA expression of 23

of them was statistically significantly modulated by at least one WWTP extract (Fig. 5). The expression of the remaining genes is presented as fold change to blank in Suppl. Table S5.

Most significant changes were observed in the down-regulated genes (Fig. 5A) upon the treatment with E4, followed by E2. Notably, the expression of several cytokine transcripts, namely *IFNG*, *IL17F*, *IL22*, *TNFSF15*, cytokine receptors (*IL21R* and *IL23R*), and several typical transcription factors, such as *FOXP3* and *TBX21*, was reduced by WWTP extracts exposure. Furthermore, the expression of MAIT cell-specific transcripts, including *GZMB*, *ICOS*, *IKZF2*, and *PRF1*, was also down-regulated. These data suggest a preferred action against MAIT cell subsets especially releasing IFN-γ and IL-17. On the other hand, the up-regulation of genes induced by WWTP extracts was less pronounced (Fig. 5B). In particular, a slightly higher expression of certain cytokines (*IL6*, *IL10*, and *IL17A*), receptors (*CXCR4*, *CXCR6*), and other immune-related proteins (*CCL20*, *ZBTB16*, and *NFKBIA*) was observed. Furthermore, a higher expression of hormone receptors, namely *AHR*, *PPARA*, and *PPARG* was observed, mainly after treatment with E2 and E4, suggesting that these WWTP extracts could contain chemical mixtures and concentrations able to interfere with these receptors.

3.4 WWTP extracts modulated the activation of basophils

As all four WWTP extracts showed immunosuppressive effects on all lymphocyte subpopulations analyzed, we next assessed their immunotoxic potential to interfere with the activation of basophils, an immune cell type involved in allergic reactions (Suppl. Fig. 10). Whole blood was stimulated with anti-FcεR1α, which targets the IgE receptor, or with fMLP, a bacterial component capable of attracting and activating basophils. All WWTP extracts reduced basophil activation induced by fMLP (Suppl. Fig. 10A). We observed that fMLP-induced activation was significantly reduced in the $CD63^+$ high basophil population (Suppl. Fig. 10C). This was also reflected in the total $CD63^+$ population, which includes the $CD63^+$ high and low populations. Unlike what we observed with the lymphocytes, here the reduction was of the same magnitude for all four WWTP extracts. Conversely, with anti-FcεR1α stimulation, differences of magnitude were observed in the $CD63^+$ high population. In this case, the four WWTP extracts differentially increased the percentage of activated cells, although only E4 reached significance (Suppl. Fig. 10B). Notably, a reduction in anti-FcεR1α-induced activation was observed after treatment with E1 extract in the $CD63^+$ low and $CD63^+$ total

basophil populations. In conclusion, the different trends in modulation by WWTP extracts that we observed seem to be related to the mode of activation of these cells.

3.5. Correlation between identified chemicals and immune endpoints

Of 578 chemicals analyzed by LC-HRMS screening, 339 chemicals were identified in at least one of the four WWTP extracts E1, E2, E3 and E4 (Finckh et al., 2022a; 2022b). Concentrations of the detected chemicals are shown in the appendix (Suppl. File 1). The different categories of chemicals found in the analyzed WWTP extracts are reported in Suppl. Table S6.

In order to identify which chemicals were most implicated in the adverse immune effects caused by WWTP extracts, correlation analyses were performed with chemical concentrations and immune endpoints (Fig. 6, Suppl. Fig. 11 and 12). The analyzed immune endpoints were: lymphocyte activation (activation markers TNF-α, CD69, CD71, CD134, and CD137 for all lymphocyte subpopulations and TNF-α, IFN-γ, and CD69 for MAIT cells), gene expression (40 genes) and basophil activation (high, low and total). The correlation coefficients between lymphocyte activation and chemical concentrations are shown (Fig. 6). The names of chemicals assigned to the color bares are better visualized in Suppl. Fig. 16.

Regarding lymphocyte activation, two third of correlations with chemical concentrations were negative (toward -1, blue), meaning that higher chemical concentrations associate with reduced lymphocyte activation. Chemicals clustering to the strongest negative correlation (dark blue bar, also see Suppl. Fig. 16A) include 13 pharmaceuticals, namely three carbamazepine metabolites (2-hydroxycarbamazepine, 10,11-dihydro-10,11-dihydroxycarbamazepine and 10,11-dihydro-10-hydroxycarbamazepine), amitriptyline, azithromycin, efavirenz, loperamide, metoprolol acid, mycophenolic acid, propranolol, raloxifene, ranitidine, and ropinirole; 4 pesticides, namely 2,4-dichlorobenzoic acid, fenoxycarb, isoxaben, and thiamethoxam; 1 EDC: clobetasol propionate, and 12 chemicals belonging to other categories, like industrial chemicals mainly, namely 1,3-diphenylguanidine, 2(4-morpholinyl)benzothiazole, 4-hydroxybenzotriazole, benzothiazole, harmine, icaridin, N-cyclohexyl-2-benzothiazole-amine, octyl-methoxycinnamate, perfluoroheptanoic acid, perfluorooctanesulfonic acid, p-toluenesulfonamide, and tri-isobutylphosphate. The chemical concentrations positively correlated with lymphocyte activation (dark red bar, also see Suppl. Fig. 16B) were 9 pharmaceuticals (4-aminoantipyrine, amantadine, capecitabine, glimepiride, melperon, metoprolol, N-acetyl-4-aminoantipyrine, sulfamethazine, and telmisartan), 9 pesticides

(bendiocarb, cyproconazole, desethylterbutylazine, flufenacet, prochloraz BTS40348, spiroxamine, tebuconazole, thiacloprid, and diacloprid amide), 4 EDCs (17-beta-estradiol, hydrocortisone, medroxyprogesterone acetate, and megestrol-17-acetate), and 4 other chemicals (2-benzothiazolesulfonic acid, 4-hydroxy-1-(2-hydroxyethyl)-2,2,6,6-tetramethylpiperidine, harman, and theophyllin). However, in vitro, the observed effects of STP extracts were in almost all cases a downregulation of lymphocyte activation. This suggests that although individual chemicals were positively correlated with lymphocyte activation, in the mixture the effect was a downregulatory one, overwhelmed by the negatively correlated chemicals.

The correlation between chemical concentrations and gene expression is presented in Suppl. Fig. 11. The gene expression was measured in PBMCs treated with WWTP extracts and stimulated with *E. coli* K12 to specifically activate MAIT cells. The list of chemicals present in each color group is reported in Suppl. Fig. 17.

Cluster analyses revealed that individual chemicals strongly negatively correlating with half of the analyzed transcripts were positively correlated to the other half of the analyzed transcripts (dark blue bar, also see Suppl. Fig. 17A). In particular these chemicals comprised 30 pharmaceuticals (e.g. amitriptyline, azithromycin, bezafibrate, bisoprolol, bosentan, carbamazepine, carbamazepine metabolites, valsartan), 23 pesticides (e.g. 2,4-dichlorobenzoic acid, 2-amido-3,5,6-trichloro-4-cyanobenzenesulfonic acid, bromochlorophen, chlorothalonil-4-hydroxy, chlorotoluron, dichlorprop, dimetachlor ESA, simazine, thiabendazole), 10 EDCs (17-α-estradiol, 1H-benzotriazole, 2-phenylphenol, 3,4,5-trichlorophenol, 4-androstene-3,17-dione, clobetasol propionate, desonide, ethylparaben, medroxyprogesterone, and triclosan) and 19 chemicals belonging to other categories (e.g. 1,3-diphenylguanidine, 2(4-morpholinyl)benzothiazole, 2-(methylthio)benzothiazole, 4-hydroxybenzotriazole, 7-diethylamino-4-methylcoumarin, benzothiazole). The main transcripts involved in the negative correlations were *IFNG*, *GZMB*, *IL22*, *IKZF2*, *IL21R* (down-regulated mRNAs, Fig. 5A). Positively correlated genes were mainly *CCL20*, *ZBTB16*, and *PPARG* (up-regulated mRNAs, Fig. 5B), evidencing a positive association between higher chemical concentration and higher mRNA expression.

Chemicals that were positively correlated with gene expression (dark red bar, also see Suppl. Fig. 17B) included 16 pharmaceuticals (e.g.4-aminoantipyrine, amantadine, capecitabine, glimepiride, indometacin, melperon, metoprolol, sulfamethazine), 12 pesticides (bendiocarb,

cyproconazole, desethylterbutylazine, fenpropimorph, spiroxamine, terbuthylazine), 9 EDCs (e.g. 17α-hydroxyprogesterone, 17-β-estradiol, 4-chlorophenol, androsterone, medroxyprogesterone acetate, megestrol-17-acetate), and 8 other chemicals (e.g. 5-chlorobenzotriazole, 7-amino-4-methylcoumarin, acridine, caffeine, cyclohexylamine, isophorone diamine, theophyllin). Contrary to the chemicals in the dark blue bar, these chemicals positively correlated with e *IFNG*, *GZMB*, *IL22*, *IKZF2*, *IL21R*, and negatively with *CCL20*, *ZBTB16*, and *PPARG*.

Regarding the correlation between chemicals and basophil activation, fewer significant correlations have been observed compared to lymphocytes (Suppl. Fig. 12). The list of chemicals present in each color group is reported in Suppl. Fig. 18.

It was visible that mostly the activation of basophils via anti-FcεR1α was associated to modulation by chemicals. The main chemicals that resulted in positive correlations (red bar, also see Suppl Fig. 18B), were similar to those that were negatively correlated with both lymphocyte activation and mRNA expression. The main chemicals being negatively correlated to basophils activated by anti-FcεR1α (blue bar, also see Suppl. Fig. 18A) were similar to the ones that positively correlated to lymphocyte activation and mRNA expression. Thus, it seems that the same chemicals differently modulate the activation of certain immune cell populations.

3.5.1 Prioritized chemicals for immunomodulation

A high similarity was observed between the chemicals that correlated highly with lymphocyte activation, basophil activation and modulation of mRNA expression (Figure 17A and B).

A total of 50 chemicals strongly correlated with all the three altered immune endpoints: 29 of them negatively correlated with lymphocyte activation and gene expression and positively with basophil activation; whereas 21 chemicals positively correlated with lymphocyte activation and gene expression and negatively with basophil activation. The chemicals associated with inhibitory effects on the immune endpoints (Fig. 7A), which were considered as prioritised chemicals due to the high consistency in the three correlation analyses, are listed in Suppl. Table S5 (together with use or mechanism of action). The categories of these chemicals are reported in Fig. 7C. The 29 high concern chemicals highly correlated with the inhibitory effects on lymphocyte and basophil activation are mainly pharmaceuticals, and within them there are neuroactive (6), cardiovascular (2), antibiotics (2), antiviral (1), endocrine (1), and antihistamine (1). There is also one corticosteroid in the EDC class, 4 pesticides and 11

chemicals in other categories, mainly industrial chemicals such as perfluorinated compounds and rubber additives.

4. Discussion

A large number of chemicals have been quantified in treated effluent samples from wastewater treatment plants before discharge into rivers across European countries (Finckh et al., 2022a; 2022b). The four WWTP extracts used in the present study, differed in chemical composition and concentration (Suppl. File 1). The immunoassays were used to assess the putative adverse effects of these effluents on the human immune system, namely the modulation of the activation of different lymphocyte subpopulations, the modulation of basophil activation and finally the effect on the expression of different genes on lymphocytes, in particular MAIT cells.

Lymphocyte and basophil activation was differently affected by WWTP extracts. With regard to lymphocytes, our analysis showed differences between the four effluent extracts, and established a potency ranking for inhibition from E1 (the lowest in terms of potency) to E4 (the most potent). The WWTP extracts exerted their immune inhibitory effects already at the lowest REF tested (6.26 x), so it is highly likely that similar effects could be obtained at the real concentrations (1 x) present in the effluent. The main *in vitro* effects, e.g. inhibition of TNF-α and IFN-γ production or expression of the activation markers CD134 and CD137 were observed on $CD4^+$ and MAIT cells. The latter were found to be more susceptible when highly activated following the stimulation with *E. coli*, a specific stimulus for these cells. WWTP extracts were also able to downregulate some transcripts indicative of MAIT cells functionality, such as *GZMB, ICOS*, *PRF1*, and *IKZF2*, highlighting an inhibitory effect on MAIT cells. MAIT cells represent a bridge between the innate and adaptive immunity, acting mainly against microbial agents (Ioannidis et al., 2020). Therefore, a suppression of the activation of MAIT cells could represent a basis for an increased susceptibility to infections (Rudak et al., 2018; Hinks and Zhang, 2020). Other genes that we observed to be downregulated in our study are regularly involved in Th1 and Th17 responses, namely *IFNG, TNFSF15, TBX21, IL17F, IL22, IL21R* and *IL23R*. On the other hand, transcripts typical of the Th2 pathway, such as *IL6, IL10* and *CXCR4*, were upregulated. Lymphocytes can behave differently, based on the release of cytokines (Chiba et al., 2018). Thus, a modulation of immune responses for example towards Th2, may lead to the development of allergic diseases (Romagnani, 2004; Licona-Limón et al., 2013).

Overall, the lymphocyte activation test represents a promising effect-based analysis of mixtures of chemicals, since it allows the visualization of the effect on a wide range of immune cells and enables to distinguish the potency of the mixtures. Together with the assessment of lymphocyte functionality, also the evaluation of certain mRNAs is helpful to estimate the immunomodulatory capacity and also to observe differences between the different WWTP extracts. They have been previously associated to endocrine disruption, with sample E4 showing the highest activity on glucocorticoid and progesterone receptors (Finckh et al., 2022a). Interestingly, in our experiments on lymphocytes, E4 was able to up-regulate the expression of other hormone receptors, namely *AHR*, *PPARA*, and *PPARG*, more than the other WWTP extracts. Indeed, in E4 there are some EDCs higher concentrated than in the other WWTP extracts, like 4-androstene-3,17-dione, benzophenone-3, canrenone, ethylparaben, methylparaben, and medroxyprogesterone, which exhibited activity via one or more hormone receptors, like *AHR*, *PPAR*, and *PPARG* (Ford, 2013; Piccinni et al., 2019; Gouukon et al., 2020; Shin et al., 2020; Cha et al., 2021).

In terms of basophil activation, our study revealed less potent effects of WWTP extracts on cell activation, compared to lymphocytes. Basophils are cells of the innate immune system that are programmed for rapid response to foreign agents (Chirumbolo et al., 2018). Therefore, contrary to lymphocytes, only a short pre-incubation time with the WWTP extracts was indicated. We observed that in this case too, the modulatory effect was exerted on the more highly activated population of basophils. Stimulation of these cells with fMLP mimics bacterial invasion and the ability to respond to it appears to be reduced by pre-incubation with these chemical mixtures. In contrast, the activation of these cells was increased by WWTP extracts followed by stimulation with anti-FcεR1α, which mimics an allergic immune response. Thus, in both cases, the effect of WWTP extracts appears to be detrimental to the function of these cells.

The effect-based analysis proposed here, together with the chemical characterization, allowed the prioritization of chemicals of high concern in terms of immunomodulation. The chemical mixtures that were highly correlated with immune endpoints included both: chemicals directly involved in immune responses, such as antihistamines, and chemicals with other modes of action, such as antibiotics, neuroactive, endocrine or cardiovascular drugs, as well as pesticides and industrial chemicals. As these chemicals belong to widely used categories, their involvement in the immune system should be investigated in detail in future studies. Within pharmaceuticals, there are drugs acting on the immune system, like clobetasol propionate, mycophenolic acid and ranitidine, but there are also others acting on nervous system, endocrine

585 system, and microbiota. Due to the strict interconnection between these organs and immunity,
586 also these pharmaceutical agents could easily affect the immune system (Zefferino et al., 2021;
587 Petra et al., 2015). Of the prioritized chemicals, amitriptyline, azithromycin, efavirenz,
588 loperamide and ropinirole have already been associated to immunomodulation and mainly
589 immunosuppression, supporting our results (Heaton et al., 2022; Isozaki et al., 2022; Juárez et
590 al., 2018; Johnson et al., 2016; Lin et al., 2016). Furthermore, also cardiovascular
591 pharmaceuticals, like beta-blockers, have been previously linked to immunomodulation (Shaw
592 et al., 2009; Maisel et al., 1991). The cardiovascular pharmaceuticals on our prioritization list,
593 metoprolol acid and propranolol, have not been evaluated by now for their immunological
594 effects. From the category of pesticides, for example for fenoxycarb, which is also considered
595 to be an EDC, it has been shown that it is able to alter the immunity of the gut (Attarianfar et
596 al., 2023). For the other pesticides in the prioritization list, no or few information are present in
597 literature. Regarding industrial chemicals, it is widely known that perfluorinated compounds
598 can affect the immune system (EFSA CONTAM Panel, 2020). In our study perfluoroheptanoic
599 acid and perfluorooctanesulfonic acid were highly associated with immunomodulation,
600 supporting published data showing the interference with the two perfluorinated compounds
601 with immune development and immune phenotyping (Torres et al., 2021; Maddalon et al.,
602 2023; Weatherly et al., 2023). Furthermore, rubber additives such as benzothiazoles or widely
603 used UV-filters (e.g. octyl-methoxycinnamate) we found, are also able to influence the immune
604 response (Ferraris et al., 2020; Khan et al., 2016). Thus, there is a body of published data in
605 support of the chemicals of immunological concern identified in our study. Certainly, several
606 other chemicals contribute to the observed adverse immune effects, such as chemicals that were
607 similarly concentrated in the different WWTP extracts, and were therefore not highlighted in
608 the correlations, as well as other chemicals that were not detected. The characterization of the
609 WWTP extracts revealed the concentrations of 339 chemicals, but even more chemicals are
610 likely to be present. Therefore, mixture effects are expected that exceed the effects of the
611 individual mixture components (Escher et al., 2020). Compounds exhibiting similar modes of
612 action typically act according to concentration addition (Kumari and Kumar, 2020; Altenburger
613 et al., 2020; Kortenkamp, 2014), whereas, chemicals with different mode of actions usually act
614 according to the independent action model (Kortenkamp, 2014). Contribution of single
615 compounds to mixture toxicity can be observed at very low concentrations, even below
616 detection limits (Kortenkamp and Faust, 2018). Therefore, the ultimate immunosuppressive
617 effect observed may be due to the action of individual chemical entities, but also to the
618 synergism and antagonism between them. The characterization of the chemical content and

concentration of different agents is an important step in identifying the causal factors leading to adverse health effects in humans (Escher et al., 2017). To date, a risk assessment approach for chemical mixtures is lacking (Liew and Guo, 2022), especially in terms of immunomodulation. For this reason, we propose a combinatorial method that combines effect-based and chemical-based analysis to fill the gap in the risk assessment of mixtures. WWTP extracts are a mixture of chemicals that reflect human activities and also environmental impacts, representing so-called "real life mixtures". Their effects are relevant to the ecosystem and are becoming increasingly relevant to humans due to climate change and the need to conserve fresh water. Undoubtedly, the earth's limited water resources will lead to a global trend towards the use of reclaimed water, for example in agriculture, but the safe use of reclaimed water for human health is still in its infancy. Here, we developed specific *in vitro* assays to unravel the effect of chemical mixtures present in four European effluents of WWTP on the human immune response. We have demonstrated the ability of wastewater treatment plant effluents to adversely affect immune cell activation in vitro, which could potentially lead to adverse health outcomes, as dysregulated immune responses underlie many diseases, including infections, cancer, autoimmunity and other chronic inflammatory diseases.

5. Conclusions

In this study, we combined chemical characterization of WWTP extracts with effect-based methods, namely immunoassays together with gene expression analysis, in order to provide information on the immunomodulatory effects of the chemical mixtures in European WWTP effluents. We found several chemicals that were highly correlated with reduced immune activation, indicating that these substances may be chemicals of very high concern that should be further investigated and monitored. More broadly, we believe that our approach can contribute to the hazard identification of WWTP effluents that are highly contaminated with chemicals of concern for immunotoxicity. Our data also highlight the need for action to improve wastewater treatment to reduce the risk to the environment and human health from reuse of this water.

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Author contributions

A. Maddalon: investigation, methodology, writing - original draft preparation; **A. Pierzchalski**: supervision, methodology, reviewing; JLK: software, reviewing; **M. Bauer**: methodology, reviewing; **S. Finckh**: reviewing; WB: reviewing; **A. C. Zenclussen**: reviewing, resources; **M. Marinovich**: reviewing; **E. Corsini**: reviewing; **M. Krauss**: reviewing; **G. Herberth**: conceptualization, data curation, supervision, writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of Generative AI and AI-assisted technologies in the writing process

No generative AI or AI-assisted technologies have been used for the writing of this manuscript.

Data availability

Data will be made available on request.

Supplementary data

Appendix A.

Supplementary Figures (1-18), Supplementary Tables (S1-S7).

Appendix B.

678 Supplementary File 1 (Table 1)

679 **Appendix C.**

680 Supplementary File 2 (Tables 1-2)

681

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Figure captions

Figure 1. Modulation of $CD4^+$ (A), $CD8^+$ (B), MAIT (C), and NKT cell (D) cell activation. Expression of TNF-α, CD69, CD71, CD134, and CD137 was measured by flow cytometry after 20 h treatment of PBMCs with WWTP extracts at REF of 6.25, 12.5, 25, followed by stimulation with anti-CD3/CD28. Only activation markers responding to stimulation in the certain immune cell populations are shown. Results are presented as FC of anti-CD3/CD28 stimulated blank-treated PBMCs. The color change from white (0) to blue (1.0) to red (1.5) indicates a decrease (from 1.0 to 0), an increase (from 1.0 to 1.5), or no change (1.0) compared to blank. Data represents the mean of 5 donors. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test. Statistical significance: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 *vs* blank. (E) Representative density plots of CD69 expression in $CD4^+$ cells treated with blank or WWTP extract E3 at increasing concentrations. The same presentation for the other T cell populations is shown in Suppl. Fig. 14.

Figure 2. t-SNE analysis of $CD4^+$ cells. Expression of TNF-α, CD69, CD71, CD134, and CD137 was measured by flow cytometry after 20 h treatment of PBMCs with WWTP extracts at REF of 6.25, 12.5, 25, followed by stimulation with anti-CD3/CD28. Only data for CD4+ cells and REF 25 are shown. (A) Representative 2D t-SNE maps, representing the merged treatment and donor condition, for the expression of CD134 and CD137 showing the blank (B) and the WWTP extract (E1-E4)-treatments. $CD4^+$ population was detected using t-SNE transformation tool, and cell activation was visualized by setting a threshold level of 1000 for CD134 (pink) and of 2000 for CD137 (yellow). (B) 2D t-SNE map of all $CD4^+$ merged events showing, based on the expression levels of all activation markers and manually gated, the 17 clusters of activated cells (active clusters) and 1 inactive cluster. The red and blue dotted oval represent the highly targeted populations. The table presents the gate color, name and % of gated cells. Due to low cell numbers in some clusters and overlap, not all colors are visible in the 2D t-SNE map. (C) Heatmap with the data obtained from the % of gated cells of each donor and treatment condition. Results are expressed as FC calculated on blank-treated PBMCs anti-CD3/CD28 stimulated (1.0, blue). The color changes from white (0) to blue (1.0) to red (2.5) indicates a reduction (from 1.0 to 0), an increase (from 1.0 to 2.5), or no change (1.0) compared to blank. Violet dotted clusters belong to both red and blue dotted ovals. Data represents the mean of 5 donors. Statistical analysis was performed by paired t-test. Statistical significance: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 *vs* blank.

Figure 3. Modulation of MAIT cell activation (A). Expression of TNF-α, CD69, CD71, CD134, and CD137 was measured by flow cytometry after 20 h treatment of PBMCs with WWTP extracts at REF of 6.25, 12.5, 25, followed by stimulation with *E. coli* K12. Only activation markers responding to stimulation in this immune cell population are shown. Results are presented as FC of *E. Coli* K12 stimulated blank-treated PBMCs. The color change from white (0) to blue (1.0) to red (1.5) indicates a decrease (from 1.0 to 0), an increase (from 1.0 to 1.5), or no change (1.0) compared to blank. Data represents the mean of 5 donors. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test. Statistical significance: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 *vs* blank. (B) Representative density plots of CD69-TNF-α-co-expression in MAIT cells.

Figure 4. t-SNE analysis of MAIT cells. Expression of TNF-α, CD69, CD71, CD134, and CD137 was measured by flow cytometry after 20 h treatment of PBMCs with WWTP extracts at REF of 6.25, 12.5, 25, followed by stimulation with *E. coli* K12. Only data for MAIT cells and REF 25 are shown. (A) Representative 2D t-SNE maps, representing the merged treatment

and donor condition, for the expression of CD69 showing the blank (B) and the WWTP extracts (E1-E4)-treatments. MAIT cell population was detected using t-SNE transformation tool, and cell activation was visualized by setting a threshold level of 5000 for CD69 (blue). (B) 2D t-SNE map of all MAIT cell merged events showing, based on the expression levels of all activation markers and manually gated, 7 clusters of activated cells (active clusters) 1 low active and 1 inactive cluster. The red and green dotted oval represent the highly targeted populations. The table presents the gate color, name and % of gated cells. Due to low cell numbers in some clusters and overlap, not all colors are visible in the 2D t-SNE map. (C) Heatmap with the data obtained from the % of gated cells of each donor and treatment condition. Results are expressed as FC calculated on blank-treated PBMCs *E. coli K12* stimulated (1.0, blue). The color changes from white (0) to blue (1.0) to red (2.5) indicates a reduction (from 1.0 to 0), an increase (from 1.0 to 2.5), or no change (1.0) compared to blank. Violet dotted clusters belong to both red and blue dotted ovals. Data represents the mean of 5 donors. Statistical analysis was performed by paired t-test. Statistical significance: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 *vs* blank.

Figure 5. Modulation of mRNA expression in *E. coli*-stimulated PBMCs. PBMCs were treated (20h) with WWTP extracts at REF 25, followed by stimulation with *E. coli* K12 to specifically stimulate the MAIT cells. Data are presented as FC calculated on *E. coli* K12-stimulated blank-treated PBMCs (1.0, dotted line). The presentation is divided in transcripts that were statistically significantly down-regulated by at least one WWTP extract (A) and those statistically significantly up-regulated by at least one WWTP extract (B). Data represents the median of 5 donors (black line within each violin). Statistical analysis was performed following Welch's t-test. Statistical significance: with *p≤0.05, **p≤0.01, ***p≤0.001 *vs* blank.

Figure 6. Correlation between chemicals and immune cell activation. The heat map shows the correlation coefficients between the concentration of WWTP extract chemicals and the % of activated immune cells, and the hierarchical clustering of similarly affected immune cells (horizontal) and similarly affected WWTP extract chemicals (vertical). Expression of TNF-α, CD69, CD71, CD134, and CD137 was measured by flow cytometry after 20 h treatment of PBMCs with WWTP extracts at REF of 6.25, 12.5, 25, followed by stimulation with anti-CD3/CD28 or *E. coli* K12. Data of the % of activated $CD4^+$, $CD8^+$, MAIT, NKT, NK, B and $CD4^-CD8^-$ cells at REF 25 are shown. Color code: negative correlation (blue) to positive correlation (red). The color bars were used to group chemicals based on similar correlation coefficients with immune cell activation. The list of chemicals present in each color group is reported in Suppl. Fig. 16. The Kendall correlation coefficients and the p values are reported in Suppl. File 2.

Figure 7. Venn diagrams showing the relationships between the three sets: lymphocyte activation (blue), basophil activation (red), and gene expression (yellow). The most highly correlated chemicals, highlighted from the heatmap with the highest correlation coefficients (dark blue and red bars for Fig. 6, suppl. Fig. 11 and 12), were evaluated. (A) The amount of chemicals negatively correlated with lymphocyte activation and gene expression (dark blue bars in Suppl. Fig. 16A and 17A) and positively correlated with basophil activation (red bars in Suppl. Fig. 18B) are shown. (B) The amount of chemicals positively correlated with lymphocyte activation and gene expression (dark red bars in Suppl. Fig. 16B and 17B) and negatively correlated with basophil activation (blue bars in Suppl. Fig. 18A) are shown. The diagrams were created using BioVenn (Hulsen et al., 2008 - https://www.biovenn.nl/index.php). Pie charts of the chemical categories of the chemicals resulting from the three correlation analyses. Categories of the common chemicals resulting from Fig. 7A (C) and from Fig. 17B (D), with their percentage.